METHODS OF IDENTIFYING AND CHARACTERIZING MUTATIONS WITHIN BACTERIAL DNA GYRASE ~~AND FABI~~

BACKGROUND OF THE INVENTION

Discovery and development of an antibacterial agent is aided by the knowledge by which the compound inhibits cell growth. A common technique in elucidating this information is to isolate mutants that alter the susceptibility of the organism to the compound and identify what mutation is responsible for the phenotype. By identifying what gene the mutation lies in, or affects the expression of, one can often learn what cellular pathway the compound inhibits, what the compounds binds to to affect growth, and obtain information about how the compound binds to the target molecule.

Random mutagenesis followed by phenotypic selection, such as resistance to an antibacterial agent, has been shown to be an effective technique to establish structure-function relationships for proteins in yeasts, viruses and bacteria (Kwan T., Gros P., *Biochemistry*, 1998;37:3337-3350; Loeb D.D. et al., *Nature*, 1989;340(6232):397-400; Rolli V. et al., *Biochemistry*, 1997;36:12147-12154). The success of such experiments is determined, in part, by the randomness of the mutagenic procedure, the ability to select for mutants of interest, the numbers of mutant cells that can be generated, and the ability to identify the mutation that is responsible for the phenotype of a selected mutant. This procedure has been used to isolate mutants of genes that are cloned into plasmids or other extrachromosomal elements. While this can work in some instances, the technique is labor intensive and is complicated in cases where the strain is diploid for the gene of interest or toxic when the gene of interest is expressed from a multicopy plasmid.

Recently, Kok et al have shown that combining mutagenesis of a defined fragment by PCR with natural transformation is a way to identify mutations which abolish the function of PobR in *Acinetobacter* region (Kok R., D'Argenio D., and Ornston L.N., Combining localized PCR mutagenesis and natural transformation in direct genetic analysis of a transcriptional regulator gene, *pobR*. *J. Bac.*, 1997;179:4270-4276). This technique exploits the ability of *Acinetobacter* to take

up PCR products and, through homologous recombination, replace the chromosomal *pobR* gene with the one that was amplified by PCR. If a PCR produced *pobR* contains a mutation that results in a dysfunctional gene product and this mutation is incorporated into the chromosome, the resulting organism would not be able to convert 4-hrdroxybenzoate to a toxic metabolite and would be viable in the presence of 4-hydroxybenoate.

In the experiments detailed below we demonstrate the use of combining random mutagenesis of genes coding for known targets of antibacterial compounds with homologous recombination to generate and identify point mutations resulting in resistance to antibacterial compounds. By using error prone long-PCR with oligonucleotide primers designed to generate large (approximately 10,000 – 15,000 base pair) overlapping products encompassing the entire genetic material of an organism, the entire chromosome of an organism can be randomly mutated in defined fragments. Following transformation and homologous recombination, the ability each of these products, and subsequently each region of the chromosome, to contain a mutation that results in an altered phentoype, such as resistance to an antibacterial compound, can be examined. By comparing the DNA sequence of the region of a mutant organism corresponding to the PCR product used to generate the mutant to the analogous region of the wild-type chromosome the mutation(s) responsible for the phenotype can be identified.

PCR amplification of the entire genetic material of an organism can also be used to identify a mutation in a chromosome resulting in an altered phenotype generated by any other technique. For these experiments, the entire chromosome of the mutant organism would be segregated into overlapping 10,000 – 15,000 base pair PCR fragments. The ability of each region to restore the altered phenotype of a wild-type strain following transformation and homologous recombination could then be used to isolate the location of the mutation to a region defined by the product used to re-create an organism with the mutant phenotype. The DNA sequence of this region could then be examined from the mutant organism and compared to the analogous region of the wild-type type chromosome to identify the mutation responsible for the phenotype. This technique will be useful in identifying mutations responsible for antibacterial

resistance in spontaneous mutants and mutants generated using DNA damaging agents.

SUMMARY OF THE INVENTION

This instant invention is a method for identifying molecular targets in bacteria treated with an antibacterial compound. The method is based on creating and identifying mutations in bacteria that confer altered susceptibility to an antibacterial compound. The mutations provide valuable information about the molecular target of the compound and how the compound and target interact. The bacterial strains generated can be used to provide information that could be useful in identifying and characterizing compounds that could be used or developed for treating bacterial infections of humans, other animals and plants.

Using *Neisseria gonorrhoeae*, we subjected *gyrA* or *fabI* to site-specific and random nucleotide mutagenesis to identify mutations that conferred resistance to ciprofloxacin or diphenyl ethers, respectively. These experiments identified previously described and novel mutations associated with resistance to these compounds. These experiments also demonstrate the ability to create and identify mutations in *Neisseria gonorrhoeae* associated with resistance to antibacterial compounds by combining random mutagenesis with phenotypic selection.

The instant invention is a system that allows for the simultaneous creation and identification of mutations that confer resistance to antibacterial compounds.

This technology is for the identification, or isolation and identification, of mutations responsible for altered susceptibility of several bacteria to chemicals (or any other selectable phenotype). This invention can be used in any bacteria that can be transformed with DNA, can carryout homologous recombination and for which the genome sequence can be determined. Examples of these include, but are not limited to: *Neisseria gonorrhoeae*, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Acinetobacter*, *Escherichia coli*, *Staphylococcus aureus*,

Streptococcus pyogenes, Pseudomonas aeruginosa, Enterococcus faecalis, Enterococcus faecium, Bacillus subtilis, and *Helicobacter pylorri*.

This invention is a process for identifying and characterizing drug-target interactions comprising:

a) Generating a defined set of overlapping PCR products (about 10 kilobasepairs per product) using chromosomal DNA from *Neisseria gonorrhoeae* strain N400 as a template which, taken together, comprise the complete DNA composition of the chromosome of the organism. The PCR reactions are executed in such a way as to introduce, in a random fashion, changes in the nucleotide composition such that some of the resulting DNA fragments have one or more changes in nucleotide composition as compared to the template DNA. These changes occur nearly randomly as a function of the PCR conditions and the DNA polymerase that is used.

b) Transforming N400 with pools of the ~10 kilobasepair PCR products, corresponding to about 100 kilobasepairs of the chromosome.

c) Isolating new strains of *Neisseria gonorrhoeae* from the transformation that show altered susceptibility to a chemical. Since the PCR fragments are generated by pairs of primers that correspond to portions of the chromosome for which the DNA sequence has been determined, the mutation or mutations responsible for the altered susceptibility can be assigned to a particular region of the chromosome.

d) Transforming *Neisseria gonorrhoeae* N400 separately with each of the PCR products that comprised the pool in 'b' to identify the region of the chromosome that is flanked by one pair of primers that carries the mutation or mutations responsible for the altered susceptibility;

e) Designing new primers based on the sequence of the region defined in 'd' to generate smaller overlapping PCR products, (about 3

kilobasepairs for each PCR product) and use to amplify chromosomal DNA from the strain isolated in 'd'.

f) Transforming N400 with the PCR products from 'e' to define the approximately 2 kilobasepair or smaller region of the chromosome that has the mutation or mutations responsible for the altered susceptibility;

g) Sequencing the DNA from the approximately 2 kilobasepair or smaller region defined in 'f'.

h) Comparing the DNA sequence with DNA sequence from the same region from N400. If a single change in the order of the nucleotides is found, this change is defined as a mutation which confers altered susceptibility to the compound. If more than one change is observed, additional rounds of primer design, PCR amplification, transformation and selections are executed so that the contribution of each mutation to the phenotype can be determined.

This invention can also be used to identify mutations that confer altered susceptibility to a chemical in strains of *Neisseria gonorrhoeae* that have previously been isolated using other methods. In this case, Step 'a' above would be:

a) Generating a defined set of overlapping PCR products (about 10 kilobasepairs per product) using chromosomal DNA from a mutant strain of *Neisseria gonorrhoeae* as a template that had previously been generated and demonstrated to be more or less susceptible to a chemical than N400. The PCR products, taken together, comprise the complete DNA composition of the chromosome of the mutant organism.

Steps b-h would be identical to that described above.

Further, the invention is a process for identifying and characterizing drug-target interactions using *Neisseria gonorrhoeae* comprising:

a) mutagenizing randomly a defined region of the chromosome that may alter susceptibility to chemical compounds. This region can

encompass i) a single codon using splicing by overlapping extension with oligonucleotides degenerate at a specific codon, ii) 20 to 100 base pairs using oligonucleotide mediated site-specific mutagenesis with a degenerate oligonucleotide, or iii) an entire gene or region definined as defined in 'f' above using low-fidelity PCR;

b) introducing mutations generated from 'a' into a wild-type background in such a manner that the wild-type region is replaced by the mutant region;

c) isolating organisms with an altered phentoype such as resistance to a chemical compound;

d) sequencing and comparison of the entire region transformed in 'b' to identify the mutation or mutations responsible for the phenotypic alteration as described in step g and h above;

d) using strains or purified proteins with mutations identified in steps 'a-d' to help to understand the mechanism of action, define the binding site and/or identify resistant forms of targets of antibacterial compounds.

The invention also pertains to a process for identifying and characterizing a mechanism of action of an antibacterial compound comprising:

generating DNA fragments by polymerase chain reaction amplification of DNA from an entire genome of a bacteria under conditions that allow for mutation of the fragments;

allowing one or more of the generated DNA fragments to be incorporated into the chromosome of a bacteria by homologous recombination;

isolating the bacteria that demonstrate resistance to an antibacterial compound; and

identifying the mutation contained in the DNA fragment.

The invention also pertains to a process for identifying mutations contained in the chromosome of a bacteria that results in an identifiable phenotype comprising:

(a) generating DNA fragments by polymerase chain reaction amplification of the bacterial chromosome corresponding to regions of the bacterial chromosome which may contain a mutation;

(b) allowing one or more of the DNA fragments to be incorporated into the chromosome of a bacteria that does not display the identifiable phenotype by homologous recombination;

(c) isolating bacteria that demonstrate the identifiable phenotype; and

repeating steps a through c until a single DNA fragment less than about 10 kilobases in length is identified as being responsible for the mutation; and

identifying the mutation contained in the DNA fragment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the generation of site-specific mutants using splicing by overlapping extension.

Figure 2 shows the generation of *Neisseria gonorrhoeae* with quinolone resistant mutations in *gyrA*.

Figure 3 shows transformation efficiencies and genotypes isolated from PCR-mediated mutagenesis of *gyrA*.

Figure 4 illustrates an overview of rapid antimicrobial target elucidation.

DETAILED DESCRIPTION OF THE INVENTION

Understanding the mechanism of inhibition of antibacterial compounds is beneficial to the discovery and development of an effective antibiotic. Natural-competence and the highly recombinant nature of *Neisseria gonorrhoeae* make this organism ideal for identifying and characterizing drug-target interactions. We use *Neisseria gonorrhoeae* to demonstrate the utility of this invention, however

the system is applicable to any other bacterial species that is capable of being transformed with DNA and can carryout homologous recombination.

To demonstrate the use of *Neisseria gonorrhoeae* in identifying mutations that lead to resistance to antibacterial compounds, we mutagenized *gyrA* or *fabI* using site-, domain-, and region-specific mutagenesis. These mutations were then introduced into *Neisseria gonorrhoeae* strain N400 and mutations associated with resistance to ciprofloxacin, clinafloxacin, dihyroxydiphenyl ether (DHDPE) or triclosan were identified.

PCR products containing mutations based on previously described mutations in *gyrA* (coding for a Ser91 to Phe, and Asp95 to Gly mutation) and *parC* (coding for Ser88 to Pro, and Glu91 Lys mutation) associated with quinolone resistance (Belland R.J., Morrison S.G., Ison C., and Huang W.M., *Neisseria gonorrhoeae* acquires mutations in analogous regions of *gyrA* and *parC* in fluoroquinolone-resistant isolates. *Mol. Micro.*, 1994;14:371-380) were generated using splicing by overlapping extension (Figure 1). These mutant PCR products were then recombined onto the chromosome via transformation and strains with mutant alleles were identified by their decrease in ciprofloxacin susceptibility. These experiments resulted in the creation of strains NG-2693 (*gyrA* S91F,D95G and *parC* S88P, E91K) and NG-2709 (*gyrA* S91F, D95G).

To identify other mutations in the *gyrA* quinolone resistance determining region (QRDR) associated with quinolone resistance, we randomly mutagenized the DNA sequence corresponding to residues 88-103 of *gyrA* using site-specific mutagenesis with a degenerate oligonucleotide. These mutations were then transformed into a wild-type background where ciprofloxacin resistant mutants were selected following homologous recombination. DNA sequencing of the *gyrA* QRDR from resistant mutants confirmed that Ser91 and Asp95 are independently involved in quinolone inhibition of DNA gyrase (Belland R.J. et al, 1994; Deguchi T., Yasuda M., Nakano M., Ozeki S., Ezaki T., Saito I., and KawadoY., Quinolone-resistant *Neisseria gonorrhoeae*: Correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoisomerase IV with antimicrobial susceptibility profiles, *Antimicrob. Agents Chemother.*, 1996;40:1020-1023) and revealed several previously unreported mutations in this region which confer resistance to ciprofloxacin in *Neisseria gonorrhoeae* as

shown in Table 1. Strains generated as a result of these experiments are NG-2691, NG-2698, GC 156 and GC 158.

TABLE 1. *gyrA* Ciprofloxacin Resistant Mutations Identified by QRDR Random Mutagenesis with a Degenerate Oligonucleotide

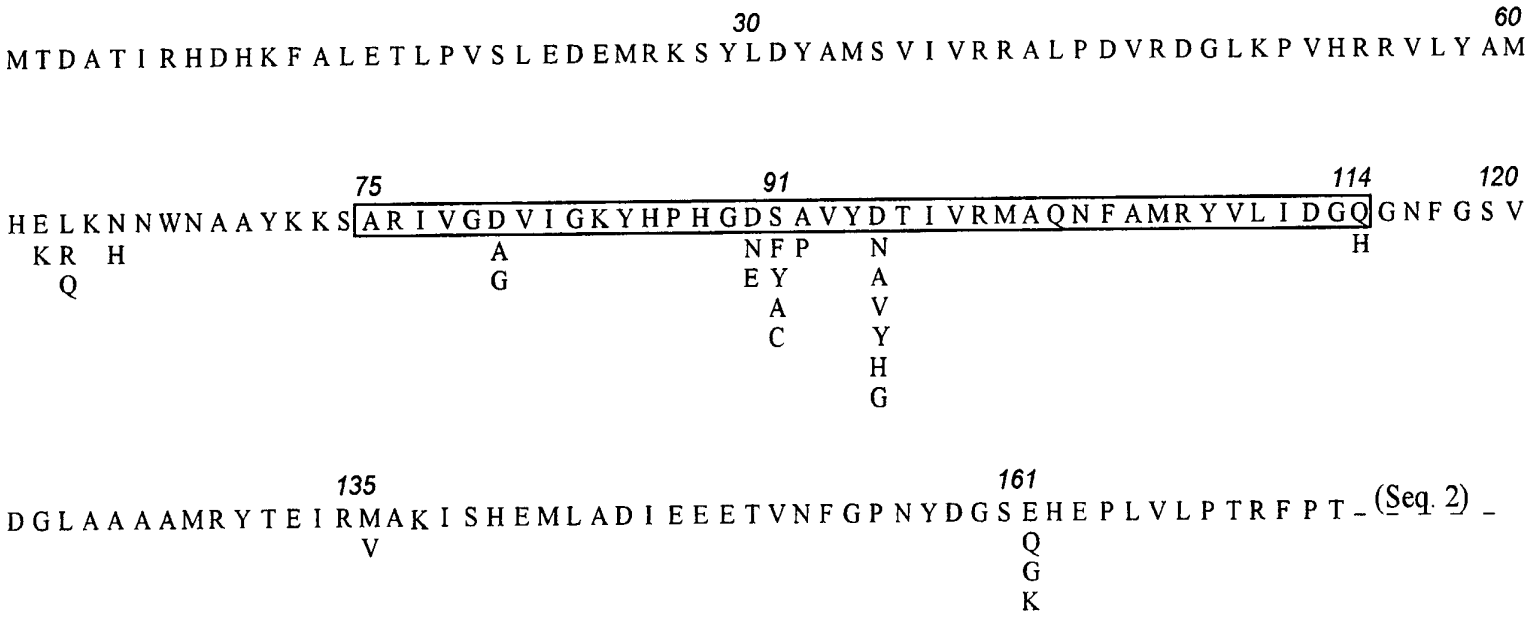
Quinolone resistant genotypes																
H	G	D E	S F C	A	V	Y	D G N H E A	T	I	V	R	M	A	Q	N	(Seq.1)

To identify other mutations leading to ciprofloxacin resistance that may not be located between residues 88 and 103 of gyrA, PCR of an 8.8 kilobasepair fragment containing *gyrA* was performed to create a pool of PCR products with random nucleotide substitutions distributed across the entire region (Kok R. et al, 1997). This pool was subsequently introduced into N400 by transformation and strains with mutations leading to ciprofloxacin resistance were isolated (Figure 3). Ciprofloxacin resistant colonies were observed at a frequency of 10^{-2} for bacteria transformed with the PCR generated library. This frequency was at least 4 orders of magnitude higher than that observed for cells that were not transformed indicating that the mutations were likely generated as a result of the PCR amplification and in the region of chromosome corresponding to the 8.8 kilobasepair PCR product used in the transformation.

To identify where in the 8.8 kilobasepair fragment the mutation responsible for the resistance was located, oligonucleotide primer pairs were designed to PCR amplify an 800 base pair product of the 5' portion of *gyrA* containing the QRDR. Each PCR product was then used to transform ciprofloxacin sensitive strains and, in all cases, was able to generate ciprofloxacin resistant colonies at high frequencies. Therefore, all ciprofloxacin resistant strains generated using an 8.8 kilobasepair random library contained a mutation in the 800 base pair region containing the 5' region of *gyrA*.

The DNA sequence corresponding to first 200 amino acids of GyrA from each ciprofloxacin resistant strain was determined to identify what mutation was responsible for the resistance. The amino acid sequence of GyrA determined from the wild-type strain is shown below with all mutations associated with ciprofloxacin resistance generated by random PCR shown underneath the respective wild-type residue. The QRDR (residues 75 – 114) is boxed. These experiments also identified both novel and previously identified mutations (Belland et al., *Mol.* Micro., 1994;14:2; Deguchi et al., *Antimicrob. Agents Chemother.*, 1995;39:561-563) associated with quinolone resistance.

Amino Acid Sequence of N400 GyrA and Quinolone Resistant Mutants



(Seq. 2)

To ensure there were no other mutations present responsible for ciprofloxacin resistance, a PCR product corresponding to the region sequenced was generated from each resistant strain. These PCR products were then transformed back into a sensitive strain, and resistant bacteria were isolated. Since high frequencies of transformation for each product were observed, and DNA sequencing confirmed that all resistant strains contained mutations identical to that seen in their respective parent, it was concluded that these mutations were solely responsible for the resistance phenotype. The strain names and codon alterations responsible for the amino acid substitutions generated by all of the *gyrA* mutagensis procedures are summarized in Table 2.

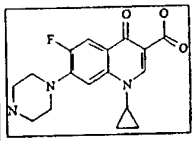
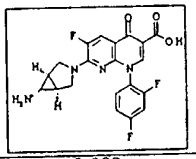
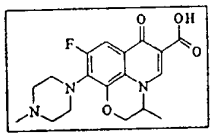
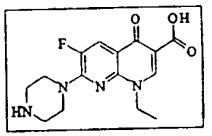
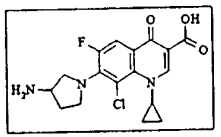
Deposits to meet the requirements of the Budapest Treaty for the purposes of patent procedure have been made. ATCC numbers will be provided.

Table 2: *Neisseria gonorrhoeae* strains

Strain Name	Parent	Genotype	Codon alteration	Phenotype for selection
NG-2707	N400	*gyrA* E62K	GAG-AAG	Clinafloxacin resistant
GC318	N400	*gyrA* L63Q	CTG-CAG	Clinafloxacin resistant
NG-2721	N400	*gyrA* L63R	CTG-CGG	Clinafloxacin resistant
NG-2711	N400	*gyrA* N65H	AAT-CAT	Clinafloxacin resistant
NG-2706	N400	*gyrA* D80G	GAC-GGC	Clinafloxacin resistant
NG-2717	N400	*gyrA* D80A	GAC-GCC	Clinafloxacin resistant
NG-2687	N400	*gyrA* S91F	TCC-TTC	Clinafloxacin resistant
GC158	N400	*gyrA* S91C	TCC-TGC	Clinafloxacin resistant
NG-2690	N400	*gyrA* S91A	TCC-GCC	Clinafloxacin resistant
GC219	N400	*gyrA* A92P	GCA-TCA	Clinafloxacin resistant
GC291	N400	*gyrA* D95G	GAC-GGC	Clinafloxacin resistant
NG-2691	N400	*gyrA* D95A	GAC-GCC	Clinafloxacin resistant
NG-2720	N400	*gyrA* D95V	GAC-GGC	Clinafloxacin resistant
NG-2723	N400	*gyrA* D95Y	GAC-TAC	Clinafloxacin resistant
GC156	N400	*gyrA* D95E	GAC-GAG	Clinafloxacin resistant
NG-2698	N400	*gyrA* D95H	GAC-CAC	Clinafloxacin resistant
NG-2709	N400	*gyrA* S91F,D95G	TCC-TTC, GAC-GGC	Clinafloxacin resistant
NG-2716	N400	*gyrA* Q114H	CAG-CAT	Clinafloxacin resistant
NG-2719	N400	*gyrA* M135V	ATG-GTG	Clinafloxacin resistant
NG-2712	N400	*gyrA* E161G	GAA-GGA	Clinafloxacin resistant
NG-2669	N400	*fabI* I15V	ATT-GTT	DHDPE resistant
NG-2654	N400	*fabI* I20T	ATC-ACC	DHDPE resistant
NG-2651	N400	*fabI* G23S	GGC-AGC	DHDPE resistant
NG-2670	N400	*fabI* A25V	GCC-GTC	DHDPE resistant
NG-2660	N400	*fabI* M51T	ATG-ACG	DHDPE resistant
NG-2641	N400	*fabI* S91T	TCC-ATC	DHDPE resistant
NG-2639	N400	*fabI* D86D, G93A	GAC-GAT, GGC-GCG	DHDPE resistant
NG-2638	N400	*fabI* G93S	GGC-AGC	DHDPE resistant
NG-2640	N400	*fabI* G93C	GGC-TGC	DHDPE resistant
NG-2648	N400	*fabI* G93V	GGC-GTC	DHDPE resistant
NG-2657	N400	*fabI* A95T	GCG-ACG	DHDPE resistant
NG-2656	N400	*fabI* A99G	GCC-GGC	DHDPE resistant
NG-2653	N400	*fabI* F104V	TTC-GTC	DHDPE resistant
NG-2658	N400	*fabI* L105H	CTC-CAC	DHDPE resistant
NG-2663	N400	*fabI* A144V	GCC-GTC	DHDPE resistant
NG-2642	N400	*fabI* Y147H	TAC-CAC	DHDPE resistant
NG-2671	N400	*fabI* G149A	GGC-GCC	DHDPE resistant
NG-2652	N400	*fabI* V159A	GTG-GCG	DHDPE resistant
NG-2661	N400	*fabI* M160I	ATG-ATA	DHDPE resistant
NG-2644	N400	*fabI* M162V	ATG-GTG	DHDPE resistant
NG-2667	N400	*fabI* I193V, Q5Q	CAA-CAG, ATC-GTC	DHDPE resistant
NG-2665	N400	*fabI* I193N	ATC-AAC	DHDPE resistant
NG-2655	N400	*fabI* T195S	ACG-TCG	DHDPE resistant
NG-2643	N400	*fabI* I201V	ATC-GTC	DHDPE resistant
NG-2666	N400	*fabI* D203V	GAT-GTT	DHDPE resistant
NG-2664	N400	*fabI* D203Y	GAT-TAT	DHDPE resistant
NG-2647	N400	*fabI* F204A	TTC-GCG	DHDPE resistant
NG-2646	N400	*fabI* F204L	TTC-TTG	DHDPE resistant
NG-2650	N400	*fabI* F204S	TTC-TCC	DHDPE resistant
NG-2649	N400	*fabI* F204I	TTC-ATC	DHDPE resistant
NG-2645	N400	*fabI* F204H	TTC-CAC	DHDPE resistant
NG-2659	N400	*fabI* A212T	GCC-ACC	DHDPE resistant
NG-2662	N400	*fabI* A212V	GCC-GTC	DHDPE resistant
NG-2672	N400	*fabI* Y247N	TAT-AAT	Triclosan resistant

To evaluate phenotypes of some of these mutants, we determined the minimum inhibitory concentrations (MICs) of a panel of antibacterial compounds. By comparing the effect these mutations have on the efficacy of both the antibacterial compound used to generate the resistant strain and related compounds, one can predict how effective these compounds will be against existing resistant strains and determine whether compounds are inhibiting cell growth through similar mechanisms. For instance, Table 3 demonstrates that ciprofloxacin, clinafloxacin, enoxacin, trovafloxacin and ofloxacin all inhibit cell growth by affecting DNA gyrase because single point mutations in *gyrA* confer resistance to all these compounds. Although ciprofloxacin, trovafloxacin and clinafloxacin show similar activities against the wild-type strain, the efficacy of clinafloxacin is more than ten times better against the high-level quinolone resistant strain (GC19). This information is very important in determining the efficacy of a compound when existing resistant species are known to be in present in the environment. It can also be concluded that residues 93 and 100 are probably not critical for the interaction between quinolones and DNA gyrase in *Neisseria gonorrhoeae* since alterations at these residues have no effect on susceptibilities to the quinolones tested. It is important to note that factors affecting the intracellular concentration of compounds (efflux pumps, modifying enzymes) are controlled for in these experiments because all strains are isogenic with exception to the mutations noted, so all changes in susceptibility are a direct result of the observed amino acid alteration.

Table 3. Susceptibilities of *N. gonorrhoeae gyrA* and *parC* mutants to a panel of quinolones

			Minimum Inhibitory Concentration * (μg/ml)					
			Ciprofloxacin	Trovafloxacin	Ofloxacin	Enoxacin	Clinafloxacin	Erythromycin
								
Strain	*gyrA* genotype	*parC* genotype						
N400	WT	WT	0.002	0.002	0.008	.06-.12	0.001	4
GC8	S91F,D95G	WT	0.06	0.03	0.25	4	0.03	2
GC19	S91F,D95G	S88P,E91K	4	4	8	64	0.25	4
GC23	WT	S88P,E91K	0.002	0.002	0.008	.06-.12	0.001	2
GC10	S91A	WT	0.008	0.008	0.03	1	0.004	2
GC158	S91F	WT	0.03	0.015	0.12	2	0.008	2
GC155	S91C	WT	0.004	0.004	0.015	.25-.5	0.002	4
GC11	D95A	WT	0.008	0.008	0.03	1	0.004	4
GC56	D95G	WT	0.015	0.008	0.06	2	0.008	2
GC59	D95H	WT	0.015	0.008	0.06	2	0.008	2
GC85	V93A	WT	0.002	0.004	0.008	.06-.12	0.002	2-4
GC87	M100A	WT	0.002	.002-.004	0.015	0.12	0.002	4

*As determined using microdilution susceptibility tests

These experiments have identified resistance mutations at the majority of residues that have been associated with quinolone resistance (a field studied for more than 30 years) from gram-negative and gram-positive bacteria. From this it can be expected that novel mutations observed in the *Neisseria gonorrhoeae gyrA* gene can also be expected to confer resistance in analogous regions of type-II topoisomerases in bacteria with at least 30% of the residues identical to the *Neisseria gonorrhoeae* GyrA QRDR (residues 75-114 of GyrA). Also, a new region of the A-subunit of *Neisseria gonorrhoeae* DNA Gyrase located near the QRDR, based on the *E. coli* crystal model, involved in quinolone resistance has been identified. The region partially comprises residues 62-65 and 161 of the Neisseria GyrA protein. The precise mechanism by which these mutations confer resistance to quinolones is currently unknown, but elucidating this information may provide valuable information for developing new inhibitors of type-II topoisomerases and defining the binding site(s) for quinolones. Since bacterial strains carrying amino acid changes at these positions reveal information about the function of the enzyme and its interaction with inhibitors, they can be used to identify and characterize compounds for use in treating bacterial infections.

In a similar series of experiments, strains of *Neisseria gonorrhoeae* that are less susceptible to the chemical dihydroxydiphenylether (DHDPE) and related compounds, including the commercially used antibacterial compound triclosan, were isolated by PCR amplifying the *fabI* gene from *Neisseria gonorrhoeae* strain N400. The PCR products were used to transform *Neisseria gonorrhoeae* strain N400 and strains that were less susceptible to DHDPE or related compounds were isolated. The *fabI* gene from each of the resulting strains was PCR amplified from chromosomal DNA and the sequence of the DNA determined. The alignment below shows the amino acid sequence of the wild-type FabI protein with all mutations that have been identified using PCR-mediated mutagenesis in *Neisseria gonorrhoeae* below their respective wild-type residue. These mutations are located across the entire gene and are concentrated at residues located in close proximity to the active site as predicted by the crystallographic structure of the *E. coli* enzyme.

Amino Acid Sequence of FabI and DHDPE or Triclosan Resistant Mutations

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                                                          30                                                        60
M G F L Q G K K I L I T G M I S E R S I A Y G I A K A C R E Q G A E L A F T Y V V D K L E E R V R K M A A E L D S E L V
                            V         T     S   V                                                   T

                                                          90                                                        120
F R C D V A S D D E I N Q V F A D L G K H W D G L D G L V H S I G F A P K E A L S G D F L D S I S R E A F N T A H E I S
                                                            T   C   V       G         L H
                                                                S
                                                                V

                                                          150                                                       180
A Y S L P A L A K A A R P M M R G R N S A I V A L S Y L G A V R A I P N Y N V M G M A K A S L E A G I R F T A A C L G K
                                              V     H                       A I   V

                                                          210                                                       240
E G I R C N G I S A G P I K T L A A S G I A D F G K L L G H V A A H N P L R R N V T I E E V G N T A A F L L S D L S S G
                        N   S           V   Y S                 T
                        V                   V L                 V
                                              I
                                              V

                                        261
I T G E I T Y V D G G Y S I N A L S T E G
                      N
```

(Seq. 3)

The strains and codon substitutions that resulted in an altered FabI amino acid sequence are shown in Table 2. These strains help understand the mechanism of the enzyme and how inhibitors of the enzyme function. Thus, they are useful in the discovery of chemicals that can be used to treat bacterial infections. Mutations at analogous codons in other bacteria that have a *fabI* gene that is similar in sequence to the *Neisseria gonorrhoeae fabI*, including, but not limited to *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium tuberculosis* would be expected to alter the susceptibility of these proteins to related inhibitors. In fact, mutations at all residues previously shown to confer resistance to triclosan or DHDPE in *E. coli* have been observed using this technique in *Neisseria gonorrhoeae* (McMurry et al, *Nature* 1998 394:531-2; Heath et al, *J. Biol, Chem.* 1999 274: 11110-11114). These mutant strains and purified proteins would be useful in discovering new chemicals for treating bacterial infections.

The previous experiments demonstrate that by using permutations of existing technologies to randomly mutate a fragment of DNA and using this DNA fragment to replace the analogous wild-type region of a bacterial chromosome it is possible to isolate strains that are resistant to chemical agents. The instant invention is an expansion of these mutagenic procedures to the entire chromosome of an organism allowing for the 1) simultaneous generation and identification of; or 2) the identification of mutations that result in an altered phenotype such as altered susceptibility to a chemical compound.

The system involves generating a library of DNA fragments from defined regions of the chromosome in such a way that the fragments contain mutations. This can be accomplished by PCR amplifying the entire genome in overlapping fragments of approximately 10 kilobasepairs. The DNA fragments can represent the entire chromosome of *Neisseria gonorrhoeae* or various portions of it. The fragments are introduced into *Neisseria gonorrhoeae* and mutants that have introduced products onto the chromosome resulting in altered sensitivity to a chemical are isolated. Isolates that have a decreased susceptibility are identified and isolated based on their ability to grow in the presence of the chemical. The result of this is that the mutation is mapped to a specific region of the chromosome as defined by the DNA that lies between the primers used to

generate the library of DNA fragments used in the transformation that created mutant strain. Once the mutation has been mapped to a reasonable sized portion of the chromosome, for example less than 3 kilobasepairs, using an iterative process of primer design, PCR amplification, transformation and selection of bacteria with altered susceptibility to the chemical, the DNA from the region of the mutant that carries the mutation can be sequenced. In this manner the mutation responsible for the altered susceptibility can be identified. From this the gene or genes involved in the mechanism by which the chemical affects the growth of the bacteria are identified.

This system can also be used to identify mutations in a bacterial chromosome that have been generated by other means and result in a phenotypic alteration. Examples of this are 1) strains carrying extra-chromosomal elements that result in a detectable phenotype, such as loss of virulence, fluorescence via green fluorescence protein (GFP) or resistance to an antibacterial compound; or 2) mutant strains containing point mutations that result in resistance to antibacterial compounds with known or unknown targets. In the former case, PCR products containing the entire genome can be systematically subjected to in vitro mutagenesis where any external fragment of DNA can be randomly inserted into the PCR product using the GPS system of New England Biolabs. The resulting PCR products can then be transformed into the wild-type strain, the extra-chromosomal material recombined onto the chromosome, and mutants containing the desired phenotype identified and isolated. In the latter case, resistant mutants can be generated using chemical means such as ethylenemethane sulfonate, DNA-damaging agents such as UV irradiation, or simply by isolating spontaneous mutants that grow on plates containing a concentration of the chemical compound that prevents growth of the parent strain. Once a strain carrying the detectable phenotype has been generated, PCR of the entire chromosome of the mutant organism in defined regions can be performed and the location of the mutation identified as described above.

This invention allows one to identify genes and gene products that can be mutated and result in an altered phenotype such as changing an organism's susceptibility to a particular chemical. This can be done without any prior information about where in the chromosome such mutations would have to occur

in order to confer altered susceptibility or without any prior information about how the chemical affects the bacteria. The result from the use of this invention is that new information regarding the interaction of the compound and the bacteria can be obtained that can be used in a program to discover and develop a new antibacterial compound for treating infections of humans, other animals and plants.

This invention can be used in bacteria other than *Neisseria gonorrhoeae*. Specifically, strains of bacteria that can be transformed with DNA, by any method, that are capable of carrying out homologous recombination and for which the complete genetic sequence can be determined. Particular examples include, but are not limited to *Haemophilus influenzae*, *Streptococcus pneumonia*, *Acinetobacter*, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Enterococcus faecium* and *Bacillus subtilis*.

MATERIAL AND METHODS

Generation of Isogenic *Neisseria gonorrhoeae* Strains With Altered *gyrA* and *parC* Alleles

PCR products containing mutations in *gyrA* (serine at residue 91 to phenylalanine and aspartate at residue 95 to glycine, hereafter referred to as S91F and D95G) and *parC* (serine at position 88 to proline and glutamate at position 91 to lysine, hereafter referred to as S88P and E91K) were created using splicing by overlapping extension (SOEing) (Ho S.N., Hunt H.D., Horton R.M., Pullen J.K., Pease L.R., Site-directed mutagenesis by overlap extension using the polymerase chain reaction, *Gene*, 1989;77:51-59). All PCR reactions were performed using genomic DNA isolated from strain N400, a derivative of MS11. Construction of the strains was done in 2 steps. The first step was to generate a DNA fragment by polymerase chain reaction (PCR) containing the desired mutation. The second step was to recombine the mutations into the chromosome, resulting in the replacement of the wild type allele for the one containing the desired mutation (allele replacement).

Generation of the PCR Product Containing site-specific *gyrA* Mutations

A 480 bp *gyrA* PCR product was generated using primers A (5'-GTCCGCCATGGCAGGTTTCTCGACAAAC-3') (Seq. 4) and B (5'-CATACGGACGATGGTGCCGTAAACTGCGAAATCGCCGTGGGGGTG-3') (Seq. 5) (altered restriction sites underlined). A 570 bp *gyrA* PCR product that overlaps the other *gyrA* product was generated using primers C (5'-CACCCCCACGGCGATTTCGCAGTTTACGGCACCATCGTCCGTATG-3') (Seq. 6) and D (5-CAACTTGAATTCGTTGACCTGATAGGG-3') (Seq. 7). The resulting PCR products were purified and combined with primers A and D in a PCR reaction to produce a 1050 bp fragment (called the *gyrA* SD-FG PCR product) containing the desired *gyrA* mutations.

Generation of Quinolone Resistance Determining Region Random Library

A 800 bp PCR product containing the first 600 bp of *gyrA* from *N. gonorrhoeae* was amplified using the oligonucleotides GC gyrA 5' NcoI (5'-GTCCGCCATGGCAGGTTTCTCGACAAAC -3') (Seq. 8) and GC gyrA 3' HindIII (5-**CCCAAGCTT**GATGGTGTCGGTGAGGTTG-3') (Seq. 9) (mutant residues in bold, restriction enzymes sites underlined). The resulting fragment and pAlterEX-2 (Promega) were digested with NcoI and HindIII, and ligated to create pAlt-gyrA.

To generate a pool of random insertions isolated to the *gyrA* QRDR (coding for residues 88-103), the oligonucleotide GC gyrA-random (5'-cacggcgattccgcagtttacgacacAatcgtccgtatggcgcaaaatTTCGC-3') (Seq. 10) was synthesized by Integrated DNA Technologies (lower case nucleotides were synthesized using phosphoramidite stock solutions contaminated with 0.7% of each non-wild type phosphoramdite, underlined is destroyed XcmI site). The resulting pool of oligonucleotides contained an average of one random mutation per oligonucleotide. This 53-mer was used for site-specific mutagenesis of pAlt-gyrA per manufacturer's protocol (Altered Sites, Promega). To ensure all colonies resulting from the mutagenesis were not wild-type, a silent C to A mutation was generated in the primer (shown in bold) which destroyed a unique XcmI site. This allowed for all plasmids to be digested with XcmI to eliminate non-recombinant plasmids. All colonies (~4000) isolated from the mutagenesis reactions were pooled together to generate a collection of plasmids containing random single

base-pair substitutions in *gyrA* corresponding to residues 88-103. This random library was then used as donor DNA in transformation experiments and strains resistant to ciprofloxacin at .002 μg/mL were isolated. The first 600 base-pairs of *gyrA* was sequenced to identify mutation responsible for the resistance.

Generation of region-specific random mutagenesis via low-fidelity long PCR.

To evaluate the ability of libraries generated from low-fidelity long PCR to generate quinolone resistant *N. gonorrhoeae*, oligonucleotides gyrA-F1 (5'-TCCATCCCGACAAATTCG-3') (Seq. 11) and gyrA-B1 (5''-TTGCGGTAGTGTTCGACCAG-3') (Seq. 12) were designed to amplify an 8.8 kb fragment that contained the entire *gyrA* gene from N400, a *recA*-inducible MS11 derivative of *N. gonorrhoeae* (Tonjum et al., *Mol. Micro.*, 1995;16:451-464). This PCR product, and one generating using the resistant allele from NG-2709 (*gyrA* S91F, D95G) as template, were transformed into the parental strain via spot transformation and incubated 24 hours as described previously (Forest et al, *Mol Microbio.* 1999 31(3):743-752). Ten microliters of 10-fold serial dilutions were then plated on plain plates and plates containing 0.002 μg ciprofloxacin per milliliter. Bacteria were then incubated 40 hours at 37°C in a 5% CO_2 atmosphere, and the frequency of ciprofloxacin resistant colony forming units (CFUs) was determined by dividing the number of ciprofloxacin resistant CFU per milliliter by the total number of cells transformed per milliliter.

Identification of mutations responsible for ciprofloxacin resistance

Ciprofloxacin resistant colonies isolated by the above procedure, using PCR libraries generated from wild-type DNA, were used as template DNA for PCR reactions using the GC gyrA 5' Nco1 and gyrA 3' HindII to generate a 800 bp product containing the 5' portion of *gyrA*. These products were then used as donor DNA for another round of transformation and selection to assess whether the resistance mutation was located within this region of the chromosome. The frequencies of transformation of all products were determined and DNA sequencing of both the donor DNA and the corresponding region of the newly created ciprofloxacin resistant mutant were determined.

Generation of DHDPE resistant FabI mutants

Random mutations in *fabI* were generated as described previously (Kok et al.) using the PCR product generated with Gc7 (5'-GGAATTCCATATGCGTAT TTGAAACGTCCAATGCC-3') (Seq. 13) and Gc8 (5'-GCACCTGCAGCAATGCGG TAC-3') (Seq. 14) using 10 ng N400 genomic DNA as template. PCR reactions were performed with either Taq polymerase (GIBCO-BRL) or the XL PCR kit (Perkin-Elmer). Ten independent PCR reactions were performed using each polymerase with the following reaction mixtures: 10µl 10x buffer (supplied with enzyme), 10 ng N400 genomic DNA as template, 20 pmoles primers, 200 µM dNTP, and either 1.5 mM $MgCl_2$ (for Taq) or 2.0 mM $Mg(OAc)_2$ (for XL PCR) (100µl final volume). The 20 reactions were pooled following 35 cycles of 95°C for 15 sec, 58°C for 30 seconds and 72°C for 1 minute. The resulting PCR products were ethanol precipitated and resuspended to 0.5 µg/ml in H_20 for subsequent transformation of gonococcal strains.

N400 was transformed with mutant PCR products using either the spot transformation technique on solid media or liquid transformation as described previously. The cells were than plated on GC solid media containing 0.5, 2 or 10 µg DHDPE per ml to select for DHDPE-resistant bacteria. Isolated colonies were passaged 2 times on GC solid media to ensure homogeneity. The *fabI* alleles were PCR amplified directly from colonies using Gc7 and Gc8 and sequenced. All PCR products containing *fabI* mutations were used to transform N400 and the selection process was repeated. If the frequency of obtaining resistant mutants was at least 100-times higher than when using a PCR product generated using N400 DNA as the template it was concluded that the mutation responsible for the resistant phenotype was in *fabI*.

Generation of random library via mutagenic PCR of large regions of the chromosome

PCR primers were designed using in-house software, PRIMER, in conjunction with BIGPRIME (a modification by the Genetics Computer Group of their PRIME program to allow for products up to 25 kb). PRIMER uses the BIGPRIME program to interactively design a list of oligonucleotide pairs to

amplify DNA regions spanning the nucleic acids sequence inputted. This region may consist of all or part of an organisms genetic material. Oligonucleotides were designed to identify primer pairs based on the following criteria; i) their ability to generate large PCR products up to 20 kilobase pairs in length, ii) the resulting products would contain at least one copy of the GC uptake sequence, and iii) each product contained at least 300 base pairs of overlapping sequence with adjacent products. The result of this process is a list of primer pairs generated that will result in the PCR amplification of the region inputted with products that overlap each other at least 300 base pairs, contain the uptake sequence, and ideally are 10 kilobase pairs in length.

Ten independent PCR products from each primer pair were generated using the Gene-Amp XL PCR kit (Perkin-Elmer) as described by the manufacturer with the following specifications: 100 μL reactions with 30 pmol of each primer pair, 10 ng of FA-1090 chromosomal DNA, 200 μM dNTP, 1 mM $Mg(OAc)_2$, and standard kit buffers. Each reaction was subjected to 35 cycles of amplification on a PE 9600 thermocycler, and PCR amplification was evaluated using agarose gel electrophoresis. The 10 analogous reactions were pooled to create a library of diverse random point mutations covering 8-12 kb of a defined portion of the *N. gonorrhoeae* chromosome.

To confirm PCR products corresponded to the region of the chromosome predicted to be amplified, restriction digest analysis was performed. Pools of PCR products were subjected to restriction digests using EcoRI or HindIII and the fragments were separated using agarose gel electrophoresis. The fragment sizes were compared to predicted digests of the identical region based on the FA-1090 sequence. Primers that were unable generate a or which resulted in patterns inconsistent with the predicted pattern were resigned using in-house software.

Generation and Isolation of Resistant Mutants

PCR products from 12 adjacent regions of the chromosome were then pooled (representing about 100 kb) and introduced into a wild-type strain by transformation (Zhang et al., *PNAS*, 1992;89:5366-5370). Briefly, 5 μg (1 μg/mL) of each pool was spotted onto a plate containing freshly streaked *N. gonorrhoeae*,

and the cells incubated overnight to allow for uptake and recombination of the mutant PCR products. Cells from each spot were then resuspended in 150 μL GC media and 5 μL of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were used to inoculate 96-well plates containing 100 μL of GC-media supplemented with Isovitalex and an inhibitory concentration of the antibacterial agent. Following 2-4 days of incubation at 37°C with 5% CO_2, wells containing viable bacteria were streaked onto plain plates and individual colonies isolated.

Identification of Mutations Conferring Resistance

To identify the mutation responsible for the resistance phenotype, DNA from the resistant mutant was amplified in 12 independent reactions using primer pairs corresponding to the region containing the resistance mutation. These products were then used as donor DNAs in transformation experiments as described above, and the PCR product containing the resistance mutation was identified by its ability restore the resistance phenotype. By generating smaller PCR products (1-4 kb) which span the 8-12 kb PCR product conferring resistance, the transformation and selection process was repeated and the mutation mapped to a 1-2 kb fragment of DNA. The DNA sequence of this fragment was determined using fluorescence-dye sequencing on an ABI 377 and analyzed using the SEQUENCHER program (Genecodes). The resulting sequence was compared to the analogous region of wild-type DNA to identify any mutation(s).